

AD _____

Award Number: DAMD17-99-1-9057

TITLE: Role of the Spindle Checkpoint in Preventing Breast
Cancers

PRINCIPAL INVESTIGATOR: Doctor Hayley McDaid
Susan Horwitz, Ph.D.
Doctor Toshiyuki Habu
Doctor Tomohiro Matsumoto

CONTRACTING ORGANIZATION: Albert Einstein College of Medicine
Bronx, New York 10461

REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2002	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jul 01 - 30 Jun 02)	
4. TITLE AND SUBTITLE Role of the Spindle Checkpoint in Preventing Breast Cancers			5. FUNDING NUMBERS DAMD17-99-1-9057	
6. AUTHOR(S): Doctor Hayley McDaid, Susan Horwitz, Ph.D., Doctor Toshiyuki Habu, Doctor Tomohiro Matsumoto				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Albert Einstein College of Medicine Bronx, New York 10461 E*Mail: MCDAID@AECOM.YE.EDU			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES <div style="text-align: right; font-size: 2em; font-weight: bold;">20030226 037</div>				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) In most of the cancer cells, chromosomal number is unstable. It has been long speculated that genome instability may be a direct cause of human cancer including breast cancer. In order to test this hypothesis, we like to abrogate the spindle checkpoint, a major surveillance mechanism responsible for maintenance of the normal chromosome number. Human/Mouse p55CDC is a target of the checkpoint. A negative dominant mutant of p55CDC that is unable to bind to Mad2 would abrogate the checkpoint. We have generated several such p55CDC mutants that have lost the ability to interact with Mad2, but otherwise normal. Expression of these mutants in Hela cells abrogated the spindle checkpoint expectedly. In BJ cell line, they are however toxic and we failed to establish an experimental system to test if a loss of the checkpoint causes chromosome instability and neoplastic transformation. Alternatively, we propose overexpression of Cmt2, a novel Mad2-binding protein we have identified recently. Our characterization indicates that Cmt2 may directly interact with the p55CDC-Mad2 complex and promote dissociation of the complex.				
14. SUBJECT TERMS breast cancer, prevention, checkpoint				15. NUMBER OF PAGES 10
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified		20. LIMITATION OF ABSTRACT Unlimited

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
Conclusions.....	6
Appendices	6

Introduction: Abnormal karyotype is one of the characters often found in cancer cells, including breast cancer. It has been reported that about 60-80 % of the cases of breast cancer show clear evidence of an aneuploid DNA index. Furthermore, most of the solid tumor cells exhibit considerable variability in chromosome number from cell to cell in the same tumor. The abnormal karyotype found in breast cancer cells may result from failure in faithful chromosome segregation.

The spindle checkpoint, that delays the onset of sister chromatid separation if a kinetochore is not attached to spindle, plays an important role for stability of the normal chromosome number. We suspect that loss of the checkpoint may produce aneuploids which triggers carcinogenesis. To test this hypothesis, we have proposed to abolish the checkpoint and examine the phenotypes.

The delay induced by the checkpoint requires that Mad2, a component of the checkpoint, binds to its target, human p55CDC, and prevents it from promoting proteolysis prerequisite to sister chromatid separation. On the basis of this principle, we have proposed to mutate p55CDC and generate mutants of p55CDC defective in binding to Mad2. Expression of such mutants would abrogate the checkpoint in a dominant manner. In year 1, we have generated such mutants of p55CDC, to which Mad2 may not bind in the yeast two-hybrid system. In the Year 2, we have further characterized these mutants *in vivo* and found that some of these p55CDC mutants indeed cannot bind to Mad2 and abolish the function of the spindle checkpoint in Hela cells. In the Year 3 (July 2001 – June 2002), we have continued the study to characterize these mutants *in vivo*. In addition, a novel Mad2-binding protein, Cmt2, has been the subject for the study supported by this grant.

Body:

1. Expression of p55CDC mutants in BJ cell lines: We showed that three mutants of p55CDC, M4, M9 and M45, abrogate the spindle checkpoint in Hela cells (Year 2). In order to test if a loss of the checkpoint can cause chromosome instability and neoplastic transformation, these mutants have been introduced into BJ cells, a cell line considered to be normal. BJ cannot grow in an anchorage-independent manner on the soft agar media, but becomes competent to grow on it upon neoplastic transformation.

We placed each p55CDC mutant under the control of a tet-inducible system and transformed into BJ cell line. Although the same construct was efficiently transformed into the Hela cells, it was not so when BJ cell line was the host. We suspected that the low efficiency of the transformation is due to the presence of the p55CDC mutant, which is probably expressed at a low level even when the tet-inducible system is turned off.

Five transformants by the construct containing M45 were analyzed for 1) expression of the p55CDC mutant and 2) function of the spindle checkpoint. We found that three of them did not express the mutant p55CDC when the tet-inducible system was turned on. The other two transformants expressed the mutant allele. They, however, maintained the functional spindle checkpoint. On the basis of these results, we concluded that a very low level of the expression of the mutant allele is toxic to BJ cell line. The five transformants we obtained either carry a secondary mutation which suppresses the mutant allele or do not express the mutant allele.

2. Characterization of a novel Mad2-binding protein, Cmt2: A novel Mad2-binding

protein, Cmt2, was identified through a screen by the yeast two-hybrid assay (Year 2). In Year 3, we obtained the results that suggested that Cmt2 may interact with Mad2 for silencing the spindle checkpoint.

2-1. Cmt2-Mad2 complex: We determined a specific stage of the cell cycle at which Cmt2 forms a complex with Mad2. Hela cells were synchronized by double thymidine block & release and cell extracts were processed for immuno-precipitation with the antibody to Mad2. At early mitosis, most of Mad2 formed a complex with p55CDC (Figure 1, IP: α -MAD2 probed with α -55CDC, lanes 8-10). A minor fraction of Cmt2 formed a complex with Mad2. In mid-mitosis, Mad2 no longer formed the complex with p55CDC. Coincidentally, the majority of Mad2 bound to Cmt2 (Figure 1, IP: α -MAD2 probed with α -CMT2, lanes 10-12). The result indicates that p55CDC that is not in the complex with Mad2 exists around mid-mitosis and that this form of p55CDC probably is an active form which promotes APC-dependent ubiquitination. The result indicates that there are two different protein complexes with Mad2. The one, Mad2-p55CDC, is formed and disassembled first. The formation of the Mad2-Cmt2 complex follows and becomes prominent in mid-mitosis.

2-2. Overexpression of Cmt2: We speculated that Mad2 switches its binding partner from p55CDC to Cmt2 in mid-mitosis. Cmt2 may interact with the p55CDC-Mad2 complex and disassemble it in mid-mitosis. To test this possibility, Cmt2 was overexpressed in Hela cells arrested by the checkpoint. The CMT2 gene, which was placed under the tet-inducible system was integrated in the genome of Hela. Upon addition of Dox (doxycyclin, analog of tetracyclin), the system would allow over-expression of Cmt2. Transfected Hela cells were blocked by thymidine and released into media that contained nocodazole.

When Dox was not added (no overexpression of Cmt2), the mitotic index, (percentage of the nonadherent pseudo-mitotic cells in population) was approximately 60 %, 14 hours after release from the thymidine block (Figure 2B). The mitotic index continued to increase (up to 80 %) if they were incubated further with nocodazole. The levels of human securin and p55CDC, both of which normally drop in mid-mitosis, remained stable under this condition. Thus, the cells were tightly arrested in the presence of nocodazole (Figure 2C).

If we added Dox 14 hours after release from the thymidine block, Cmt2 was induced and became detectable within 3-4 hours (Figure 2D). Upon the addition of Dox, the mitotic index dramatically dropped (Figure 5B). The levels of p55CDC and securin also dropped around 8 hours after the addition of Dox (Figure 2D). We concluded that induction of Cmt2 abrogates the spindle checkpoint and overcomes the mitotic arrest caused by nocodazole. The overexpression of Cmt2 probably affects the spindle checkpoint in a direct manner since the p55CDC-Mad2 complex was disassembled after the induction of Cmt2 (Figure 2E). FACS analysis demonstrated that, if Cmt2 was not induced, most of the cells contained 4N content 24 hours after release from the thymidine block, indicating that they were arrested in G2 or mitosis (Figure 2F, 24 hours, -Dox). If they were incubated with nocodazole further, cells with less than 2N content (48 hours, -Dox) appeared. This hypodiploids probably suggest that cell death was induced due to

prolonged arrest by nocodazole. Notably, overexpression of Cmt2 resulted in appearance of cells with 8N content (Figure 2E, 24 and 48 hours, +Dox), indicating that the next round of DNA synthesis was completed without the previous chromosome segregation in these cells. This phenotype is typical in cells without the functional spindle checkpoint. We examined 11 independent cell lines in which the inducible CMT2 construct was integrated and found that they all consistently exhibited this phenotype.

Observation of the cell/chromosome morphology indicated that overexpression of Cmt2 resulted in extensive abnormality in chromosome appearance. Most of the cells (~80 %) exhibited nuclei which were larger than those of normal mitotic cells (Figure 3A). Also they occasionally contained multiple nuclei (Figure 3B). In addition, nuclear mass was partially and unequally segregating in some cells (20 %, (Figures 3C and D).

Key Research Accomplishment:

- 1) The original tasks 5 - 14 were to characterize the mutant p55CDC in the animal model system. By using BJ cell line, we can avoid the animal experiments to test if the mutant p55CDC causes chromosome instability and neoplastic transformation. In the Year 3, we have, however found that BJ cell line is much sensitive to the expression of mutant p55CDC.
- 2) We found that overexpression of Cmt2 inactivates the spindle checkpoint.

Reportable Outcomes:

Toshiyuki Habu, Sang Hoon Kim, Jasminder Weinstein and Tomohiro Matsumoto. Identification of a Mad2-Binding Protein, Cmt2, and its Role in Mitosis. (EMBO J. under revision)

Conclusion:

1. BJ cell line is much sensitive to the mutant p55CDC and the original strategy to abrogate the spindle checkpoint by p55CDC mutant cannot be used. We should consider another inducible system which tightly regulates the expression.
2. Through characterization of Cmt2, a novel Mad2-binding protein, we found that Cmt2 abrogates the checkpoint when it is overexpressed at a level 10 to 20-fold higher than the native locus. This method can be used as an alternative of the mutant p55CDC.

Appendix: Figure 1, 2 and 3 are attached.

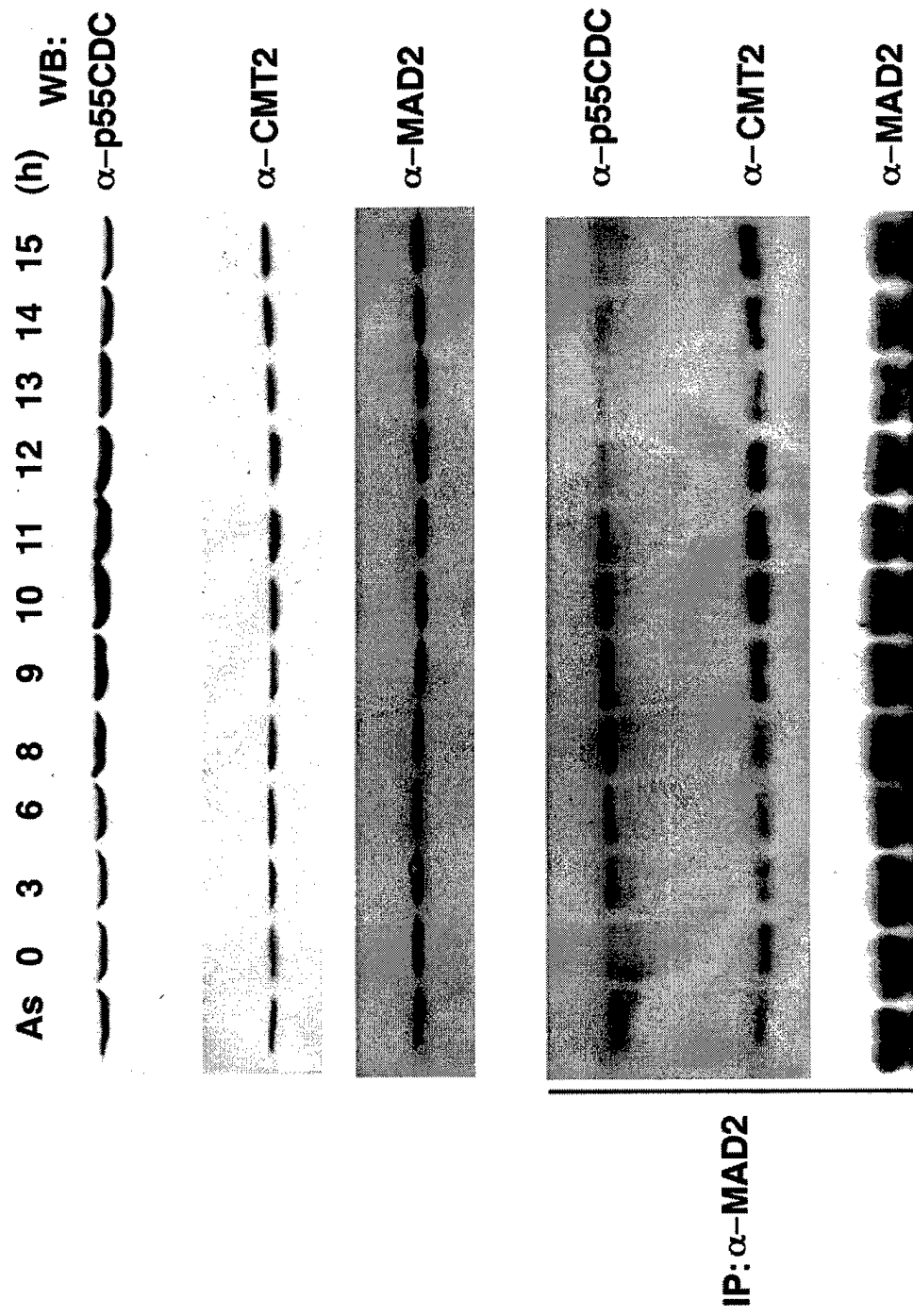


Figure 1

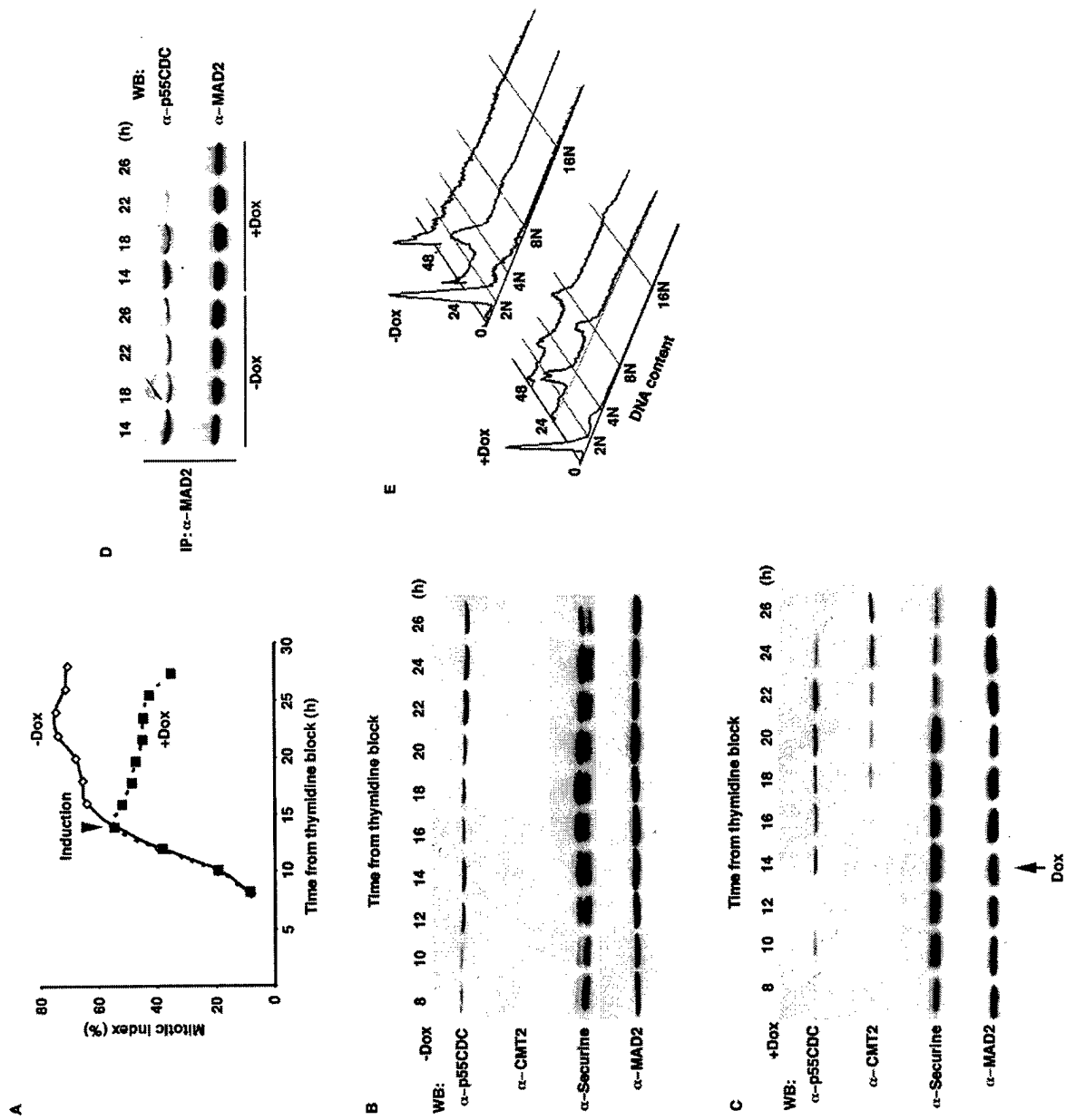
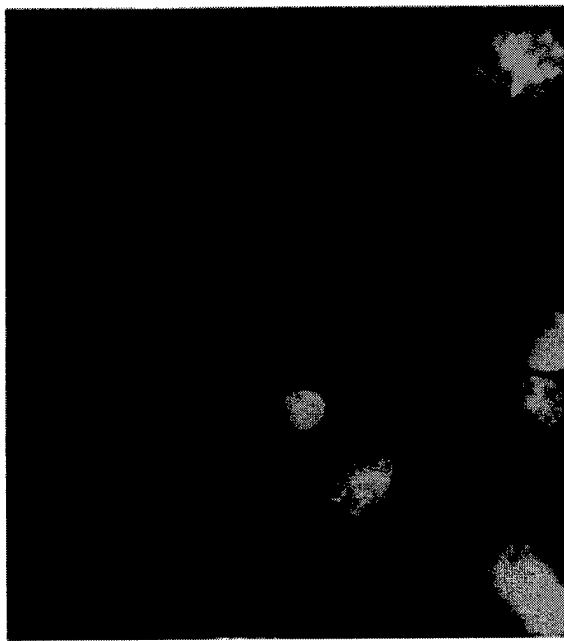
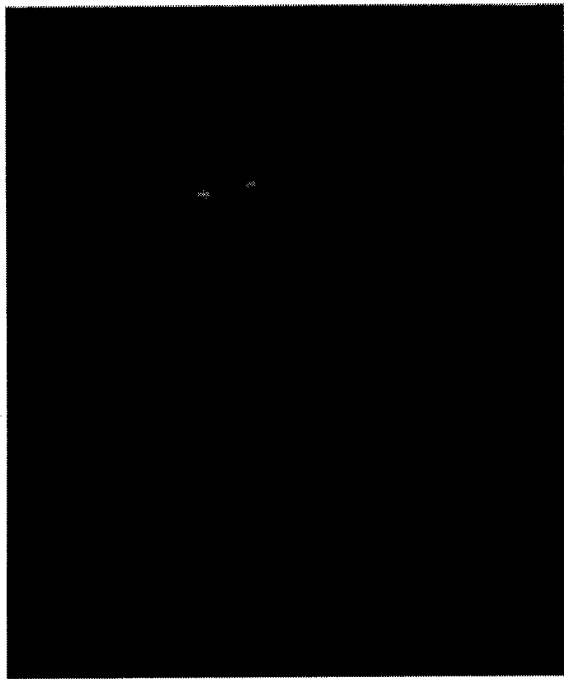


Figure 2

A



B



C



D



E



Figure 3

Legends for Figures

Figure 1: Cell cycle analysis. Cell extracts were prepared at indicated time points after the release from double thymidine block and processed for western blot (upper 3 panels) or immunoprecipitation with the antibody to Mad2 followed by western blot (lower three panels). Extracts prepared from an asynchronous culture (AS) were also examined in the same way.

Figure 2. Overexpression of Cmt2

(A) HeLa cells in which Cmt2 was inducible by Dox were arrested by double thymidine (DT) block and released at time 0. Nocodazole was added at 8 hours after the release and mitotic index was measured microscopically.

(B, -Dox) and (C, +Dox) Extracts were prepared at each time point and processed for western blot. For detection of p55CDC and Cmt2, ten μ g of total proteins were run on SDS-PAGE. Note that the endogenous Cmt2 was not detectable under this condition. Thirty μ g of total proteins for detection of securin and 50 μ g for Mad2 were used.

(D) Extracts were prepared at each time point for immunoprecipitation with the antibody to Mad2 followed by western blot.

(E) FACS analysis.

Figure 3.

The cells prepared as Figure 2 were grown for 28 hours after the release from thymidine block and stained with DAPI (for DNA) and FITC-conjugated phalloidine (for cell shape). Induction of Cmt2 in the cells arrested by nocodazole resulted in a forced exit from mitosis. The majority of them exhibited larger nuclei (A) or multiple nuclei (B). A fraction of them showed a "cut"-like phenotype (C and D). If Cmt2 was not induced, the majority of the cells exhibited condensed chromosomes with round-up cell shape, a typical phenotype of prometaphase arrest (not shown). Interphase nuclei of normal HeLa cells at late G2 are shown for comparison (E).